JANUARY 1, 1993
VOLUME 90
NUMBER 1

Proceedings
OF THE
National Academy of Sciences
OF THE UNITED STATES OF AMERICA

Proceedings
OF THE
National Academy of Sciences
OF THE UNITED STATES OF AMERICA

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PRINTED IN THE USA

Second class postage paid at Washington, DC, and at additional mailing offices.

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA (ISSN-0027-8424) *is published semimonthly by* THE NATIONAL ACADEMY OF SCIENCES, *2101 Constitution Avenue, Washington, DC 20418.*

POSTMASTER: Send address changes to: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 2101 Constitution Ave., Washington, DC 20418.

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 123–127, January 1993
Biochemistry

Signal transduction mediated by growth hormone receptor and its chimeric molecules with the granulocyte colony-stimulating factor receptor

(cytokine receptor/exon swapping/growth signal/recombinant PCR/receptor dimerization)

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Communicated by Charles Weissmann, September 28, 1992

ABSTRACT **The granulocyte colony-stimulating factor receptor (G-CSF-R) and growth hormone receptor (GH-R) belong to the cytokine receptor family and have some similarity in the cytokine receptor-homologous (CRH) domain of the extracellular region. Among members of this family, the G-CSF-R and GH-R seem to function as homodimers. Previously, we showed that mouse myeloid precursor FDC-P1 cells expressing the G-CSF-R can respond to G-CSF for growth. Here we show that the GH-R can also transduce the growth signal in FDC-P1 cells in the range 10 pM–100 nM GH. At a higher concentration of GH, GH did not promote the growth of the transformant cells. A series of chimeric receptor cDNAs between the G-CSF-R and GH-R cDNAs was constructed by exon swapping and was expressed in FDC-P1 cells. A ligand-binding assay with transformants expressing chimeric receptors indicated that the entire CRH domain is necessary for specific binding of the ligand. Although the transmembrane and cytoplasmic regions of the G-CSF-R and GH-R have no apparent similarity, these regions were interchangeable, resulting in growth-signal transduction in FDC-P1 cells.**

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that regulates proliferation and differentiation of the precursor cells of neutrophilic granulocytes (1–3). The G-CSF receptor (G-CSF-R) expressed in cells restricted to the neutrophilic granulocyte lineage was purified from mouse myeloid leukemia NFS-60 cells (4), and cDNAs for murine and human G-CSF-R were isolated (5, 6). The mouse G-CSF-R consists of 812 amino acids with a single transmembrane region and contains an immunoglobulin-like domain, a cytokine receptor-homologous (CRH) domain, and three fibronectin type III domains. The CRH domain of the G-CSF-R has significant homology with the extracellular regions of cytokine receptors (7), especially with the prolactin receptor and growth hormone receptor (GH-R) (5). Since the recombinant G-CSF-R expressed in various mammalian cell lines and the dimeric form of the purified G-CSF-R can bind G-CSF with high affinity, the G-CSF-R seems to function as a homodimer (8). Recently, we showed that exogenously expressed G-CSF-R can transduce the growth signal in mouse FDC-P1 and BAF-B03 cell lines (9). Furthermore, mutational analysis of the G-CSF-R indicated that the CRH domain of the G-CSF-R is the ligand-binding domain and that ≈100 amino acids in its cytoplasmic region are indispensable for transducing the growth signal (9).

GH produced by the pituitary gland is a member of a polypeptide hormone family that includes GH, placental lactogen, and prolactin (10). GH has various pharmacological effects *in vivo* such as bone growth, myogenesis, lactation, and activation of macrophages (11). Various effects of GH are mediated by the interaction of GH with its receptor (GH-R) (12). Leung *et al.* (13) have isolated cDNAs for the human and rabbit GH-R. The human GH-R consists of 620 amino acids and contains a single transmembrane domain. Using the recombinant protein expressed in *Escherichia coli*, it was shown that the extracellular domain of the receptor is the binding site for the GH (14). Furthermore, x-ray diffraction and mutational analyses of complexes of the GH and the GH-R extracellular domain indicated that a single ligand molecule binds to the homodimer of the receptor (the extracellular domain) (15, 16). However, because a functional expression system for the GH-R cDNA had not yet been developed, whether ligand-induced dimerization of the GH-R is actually necessary to transduce the signal into cells remained unknown.

In this study, rabbit GH-R cDNA was introduced into the mouse FDC-P1 cell line. Transformants expressing the GH-R transduced a growth signal in response to limited concentrations of GH. A set of chimeric molecules constructed from G-CSF-R and GH-R by exon swapping also transduced the growth signal into FDC-P1 cells, suggesting a similar signal transduction mechanism of the G-CSF-R and GH-R.

MATERIALS AND METHODS

Primers Used for PCR. The following class A primers are forward primers corresponding to sequences of rabbit or human GH-R cDNA: RGHR-1, TGGGGTTCATCATGGTTTAA; RGHR-2, TCAATACAAAGAAGTCAATG; HGHR-1, TGAGGTTCATCATGGTACAA. The following class B primers carry hybrid sequences consisting of the antisense strand of mouse G-CSF-R and the GH-R: RC-1R, ATGGGAGGCTCCAATTTCACTATTTCCTCAACAGAGA; RC-2R, TCCAGTCTGATGGTGGGGGCTTCTTCACATGTGAATGGG; RC-3R, TGTTTAAGTCAGATGGATCTTCTTCACATGTGAATG; RC-4R, CAGAAGGAAGTCTTTCCTCTTTGCTGTTTAGAAAATAT; RC-5R, GGGTAACTGGAAGGTTTCCTTGAGGAGATCTGGAT; HC-2R, TCCAGTCTGATGGTGGGGGCTTCTTCACATGTAAATTGG. The class C primers (RC-1F, RC-2F, RC-3F, RC-4F, RC-5F, and HC-2F) are complementary to RC-1R, RC-2R, RC-3R, RC-4R, RC-5R, and HC-2R, respectively. The following class D primers carry the antisense strand of mouse G-CSF-R: MOR2, TGGCTTCCAGAACAGCTGCAC; MOR12, GGACCTTCGTTCTCCAGGAA; MOR15, TGGCACGCTGGAGTCCCAGA; MOR16, CATTACTCCTGGGCTGGTGG.

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; G-CSF-R, G-CSF receptor; GH, growth hormone; GH-R, GH receptor; IL-3, interleukin 3; CRH domain, cytokine receptor-homologous domain.

Plasmid Construction. Plasmid pBOSI62 carrying the full-length cDNA for mouse G-CSF-R in the pEF-BOS vector (17) has been described (9). pCIS2.sHGHR carries a cDNA coding for the human GH-R extracellular domain at the *Xho* I/*Not* I site of the pCIS vector (13). The cDNA was excised from pCIS2.sHGHR by digesting with *Xba* I and *Hpa* I and then inserted into the *Xba* I site of pEF-BOS with an *Xba* I linker (CTCTAGAG). The full-length rabbit GH-R cDNA (13) in pBluescript KS (pBS-RGHR) was transferred into pEF-BOS by a similar method and was designated pBOS-RGHR.

The cDNAs for chimeric receptors between GH-R and G-CSF-R were constructed by recombinant PCR (18) using the following primer combinations: RC1 chimera, RGHR-1, RC-1R, RC-1F, and MOR2 as class A, B, C, and D primers, respectively; RC-2 chimera, RGHR-2, RC-2R, RC-2F, and MOR12 primers; RC3 chimera, RGHR-2, RC-3R, RC-3F, and MOR15 primers; RC4 chimera, RGHR-2, RC-4R, RC-4F, and MOR15 primers; RC5 chimera, RGHR-2, RC-5R, RC-5F, and MOR16 primers; HC2 chimera, HGHR-1, HC-2R, HC-2F, and MOR12 primers.

In the primary PCR, a part of the GH-R cDNA in pBOS-RGHR or pBOSsHGHR was amplified using a class A oligonucleotide as the forward primer and a class B oligonucleotide as the reverse primer. Part of the G-CSF-R in pBOSI62 was amplified using class C and D oligonucleotides. PCR was carried out in 20 mM Tris·HCl, pH 8.3/50 mM KCl/2.5 mM $MgCl_2$/0.1 mg of gelatin per ml/50 μM each dNTP/0.5 μM primers/2 ng of template DNA/2.5 units of *Taq* polymerase (Takara Shuzo, Kyoto) in a total vol of 100 μl. The conditions for PCR were 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C for 15 cycles. Products from the primary PCR were isolated by agarose gel electrophoresis and mixed 1:1; the secondary PCR proceeded as described above using class A and D primers. The product was digested with restriction enzymes and then ligated with DNA fragments of the appropriate 5′ and 3′ portions of GH-R and G-CSF-R cDNA, respectively. The structures of the resultant hybrid cDNAs were determined by sequencing. It revealed that Cys-241 in the HC2 construct was replaced by arginine, which has no effect on binding to human GH (14). The hybrid cDNAs were inserted into pEF-BOS vector to produce pRC1–pRC5 and pHC2.

Cells and Transfection. The murine myeloid precursor cell line FDC-P1 (19) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (HyClone) and 10–20 units of recombinant mouse interleukin 3 (IL-3) per ml (4). FDC-P1 cells were transfected with plasmid DNAs by electroporation as described (9). After selection with G418 (0.5 mg/ml), transformants expressing the chimeric receptor were identified by binding of human ^{125}I-labeled GH (^{125}I-GH) (DuPont/NEN) or by Western blots using rabbit anti-G-CSF-R antibody (MR-9 antibody) (9).

Growth Factors, Cell Proliferation Assay, Binding of Growth Hormone, and Western Blotting. Mouse recombinant G-CSF and IL-3 were prepared as described (4). Human GH was provided by the manufacturing group at Genentech, while bovine GH was purchased from Seikagaku Kogyo (Tokyo).

The cell proliferation assay was carried out in 96-well microtiter plates as described (9) except that 10% fetal calf serum in RPMI 1640 medium was replaced by 10% horse serum. In brief, transformed cells were extensively washed with RPMI 1640 medium containing 10% horse serum without IL-3, and 1.5×10^4 cells (100 μl) were incubated with various concentrations of human or bovine GH at 37°C for 22 hr. Thereafter, [^{3}H]thymidine (0.5 μCi; 1 Ci = 37 GBq) was added to each well, and the cultures were further incubated for 4 hr at 37°C before harvest.

Binding of GH to the transformed cells was performed with human ^{125}I-GH as described (4). Cells expressing the hybrid receptor were analyzed by Western blotting with rabbit anti-MR9 antibody recognizing the cytoplasmic region of the G-CSF-R as described (9) except that ^{125}I-protein A (DuPont/NEN) was used instead of ^{125}I-F(ab′)$_2$ fragment of donkey anti-rabbit immunoglobulin antibody.

RESULTS

Signal Transduction by GH-R in Murine FDC-P1 Cells. To examine whether the cloned GH-R cDNA can transduce a proliferative signal, the full-length cDNA for rabbit GH-R (13) was expressed under the control of the human elongation factor 1α promoter (17). The IL-3-dependent murine myeloid precursor FDC-P1 cell line was chosen as the host since it neither expresses the GH-R nor responds to GH for growth (Fig. 1).

The expression plasmid was introduced into FDC-P1 cells together with a plasmid carrying the neomycin-resistant gene. G418-resistant cell lines were then tested for the ability to bind human ^{125}I-GH, and several clones (FD-RGHR) expressing rabbit GH-R were identified. A Scatchard analysis of the binding data indicated that these transformants bind human GH with a dissociation constant (K_d) of 280 pM (Table 1), which agrees with those of the native rabbit GH-R and the recombinant molecule expressed in COS cells (13). The number of GH-R expressed in one of the transformants was 1200 sites per cell (Table 1).

When the transformants expressing the rabbit GH-R were shifted into RPMI 1640 medium containing 10% fetal calf serum without IL-3, all transformants proliferated in the absence of IL-3. This may be due to the presence of bovine GH or a GH-like factor in fetal calf serum, which interacts with rabbit GH-R (13). In contrast, the transformants could not grow in the medium containing 10% horse serum without IL-3. Therefore, [^{3}H]thymidine incorporation was assayed in medium containing horse serum. As shown in Fig. 1, the parental FDC-P1 cells did not respond to human GH, whereas the transformants expressing the rabbit GH-R responded to GH in concentrations of 10 pM–100 nM. The half-maximal response was obtained with 200–300 pM GH, which is almost identical to the K_d value obtained by binding ^{125}I-GH to the receptor. Furthermore, GH (10 nM) supported the long-term growth of the transformants in RPMI 1640 medium containing 10% horse serum without IL-3 (data not shown). As previously observed with the HC2 chimera (see

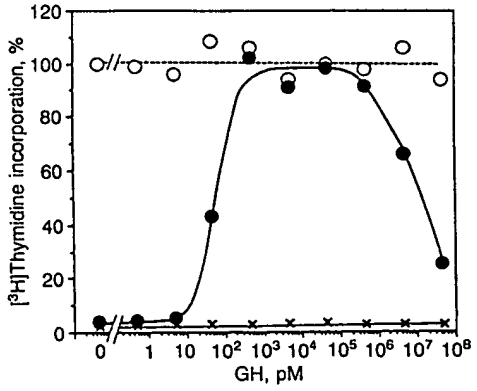

FIG. 1. GH-dependent DNA synthesis of FDC-P1 transformants expressing rabbit GH-R. Parental FDC-P1 (×) and the transformant cell line (○ and ●) were cultured with 0–50 μM human GH in the presence (○) or absence (●) of IL-3 (50 units/ml). [^{3}H]Thymidine incorporation is presented as percentage of maximum incorporation observed with excess IL-3.

Table 1. GH-binding characteristics of transformants expressing rabbit GH-R and its hybrid receptors

Cell line	K_d, pM	Sites per cell
RC1	—	No binding
HC2	130	2400
RC2	670	3000
RC3	230	6800
RC4	190	1700
RC5	180	2000
FD-RGHR	280	1200

below; ref. 20), a higher concentration of GH (>500 nM) did not promote growth of the transformants expressing the GH-R (Fig. 1). Since 50 μM GH had no inhibitory effect on the IL-3-dependent growth of the transformants, the inability of the high concentration of GH to induce their growth seems to be a specific effect on the GH-R.

Construction of Chimeric cDNAs Between GH-R and G-CSF-R. Previously, we have suggested that the G-CSF-R functions as a homodimer (9). The CRH domain of the G-CSF-R has homology with the extracellular region of the GH-R (5) and is responsible for binding of G-CSF (9). Since the GH-R also forms a homodimer (15, 16), we considered that the G-CSF-R and GH-R may function by a similar mechanism. To examine this possibility, a set of chimeric molecules of GH-R and G-CSF-R was prepared.

The G-CSF-R and GH-R both contain a ligand-binding CRH domain, a transmembrane region and a cytoplasmic region. In addition to these common regions, the G-CSF-R contains three fibronectin type III domains, but these domains are required neither for binding of G-CSF nor for signal transduction (9). Chromosomal gene analyses of GH-R and G-CSF-R showed that both receptor genes have a similar exon–intron organization (refs. 21 and 22; Y. Itoh, R.F., and S.N., unpublished results). The chimeric molecules were, therefore, constructed by exon swapping between GH-R and G-CSF-R. Hybrid primers containing sequences from GH-R and G-CSF-R cDNAs at the junction of each exon were synthesized and used to produce chimeric cDNAs by recombinant PCR as described. Fig. 2 schematically shows the structure of the chimeric receptors. In the RC1 construct, the immunoglobulin-like domain and the N-terminal half of the CRH domain of G-CSF-R were replaced with the corresponding portion of rabbit GH-R. The RC2 or HC2 chimeric receptor carries the CRH domain of rabbit or human GH-R in place of that of G-CSF-R. In the RC3 construct, the whole extracellular domain of G-CSF-R was replaced with that of rabbit GH-R. The RC4 chimeric receptor carries the extracellular domain and the transmembrane domain from rabbit GH-R and the cytoplasmic region from G-CSF-R. In the RC5 construct, the C-terminal region (encoded by the last exon) of GH-R was replaced by the C-terminal region (the last exon) of G-CSF-R.

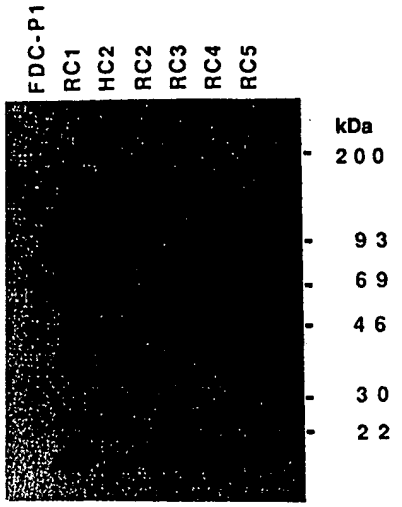

FIG. 3. Expression of chimeric receptors in FDC-P1 transformants. Cell lysates from the parental FDC-P1 and transformant cell lines (2×10^5 cells) were analyzed by Western blotting using rabbit anti-MR9 antiserum recognizing a portion of the cytoplasmic region of mouse G-CSF-R (9). Sizes of marker proteins (rainbow marker; Amersham) are indicated on the right in kDa. Positions of glycosylated mature proteins are indicated by arrows.

Expression of the Chimeric Receptors in FDC-P1 Cell Line. FDC-P1 cell transformants expressing the chimeric receptor were established as described. When these transformant clones were analyzed by immunoblotting using the MR-9 antibody recognizing the cytoplasmic region of murine G-CSF-R, both mature and partially glycosylated receptors

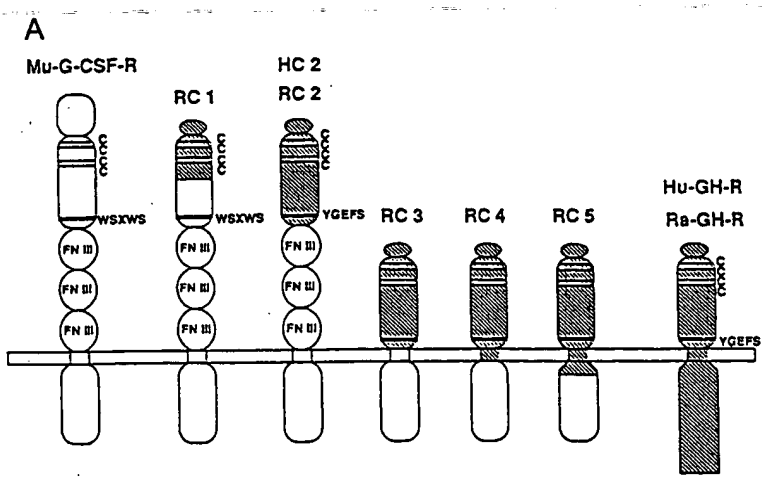

FIG. 2. Structure of GH-R and G-CSF-R chimeric receptors. (*A*) Schematic representation of G-CSF-R, GH-R, and their chimerae. Names of chimerae are indicated at the top. Open boxes represent domains derived from G-CSF-R; hatched boxes indicate those from GH-R. Thin bars indicate conserved cysteine residues; thick bars represent the WSXWS motif in G-CSF-R and the corresponding YGEFS sequence in GH-R. FN III in G-CSF-R indicates fibronectin type III domain. (*B*) Amino acid sequences at the junctions of chimerae are shown. Numbers above the line indicate amino acid number of rabbit or human GH-R (13); numbers below the line indicate amino acid number of mouse G-CSF-R (5).

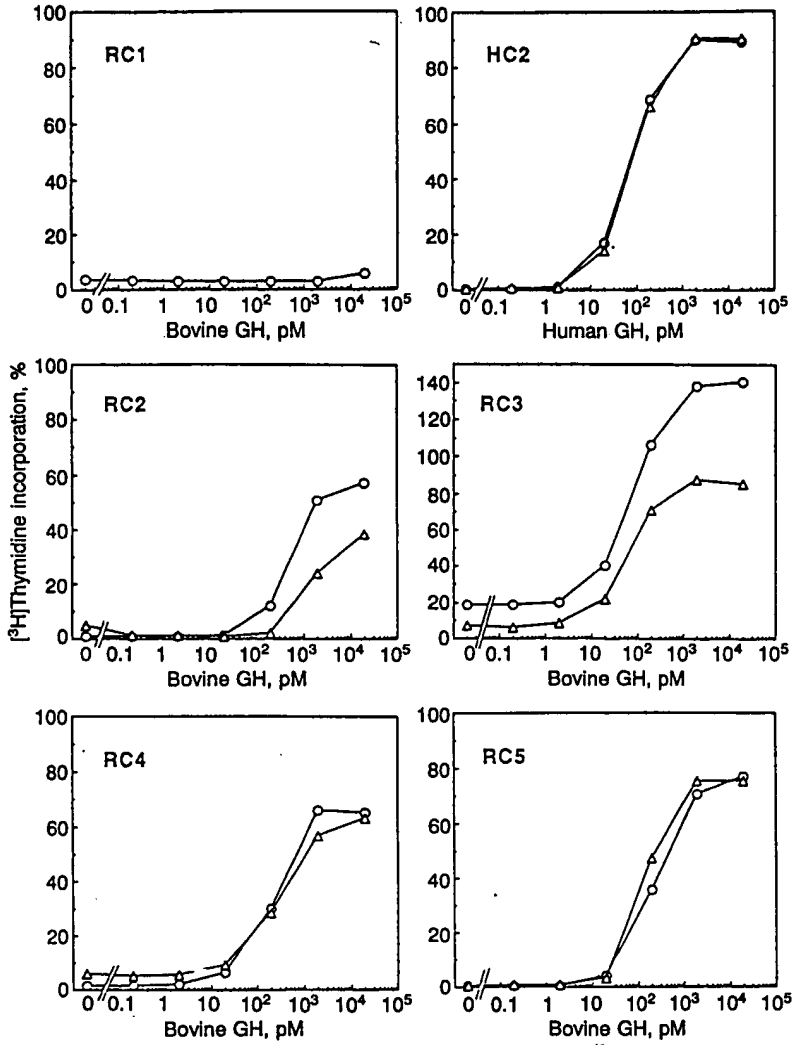

FIG. 4. GH-dependent DNA synthesis of FDC-P1 cell transformants expressing chimeric receptors. Two independent transformants (○ and △) expressing chimeric receptors were cultured in the presence of 0–20 nM bovine GH (for RC1–RC5 transformants) or human GH (for HC2 transformants), and [3H]thymidine incorporation into each cell line was assayed as described in the legend to Fig. 1.

with the predicted molecular masses were observed in all transformant clones (Fig. 3). Ligand-binding activity was assayed in these transformant clones using human ^{125}I-GH. As shown in Table 1, all transformants except those expressing RC1 bound human GH. Scatchard analyses of the binding data indicated that the HC2 and RC2–RC5 cell lines carrying the chimeric receptors express 1700–6800 binding sites for human GH per cell. The dissociation constants were around 100–300 pM for all chimeric receptors except for the RC2 construct, which had a slightly higher dissociation constant (670 pM).

Signal Transduction Mediated by Chimeric Receptors. The effect of bovine or human GH on proliferation of transformants expressing the chimeric receptors was examined by [3H]thymidine incorporation assay. As shown in Fig. 4, all transformants except RC1 responded to GH in a dose-dependent manner. The concentrations of GH showing the half-maximal responses were almost identical to the dissociation constant of human GH for each chimeric receptor (Table 1). The high concentration of GH (>100 nM) showed an inhibitory effect on the growth of the transformants expressing the HC2, RC3, RC4, or RC5 chimeric molecule, although its effect was less pronounced with the transformants expressing RC3 and RC5 chimerae (Fig. 5; ref. 22). In accord with our previous observation (9), the three fibronectin type III domains of G-CSF-R were not required to transduce the growth signal. The transformant cell lines HC2, RC2, RC3, RC4, and RC5 could proliferate for a long time in the medium containing 40 nM GH in the absence of IL-3 (data not shown). These results indicate that the transmembrane and cytoplasmic regions of GH-R and G-CSF-R are func-

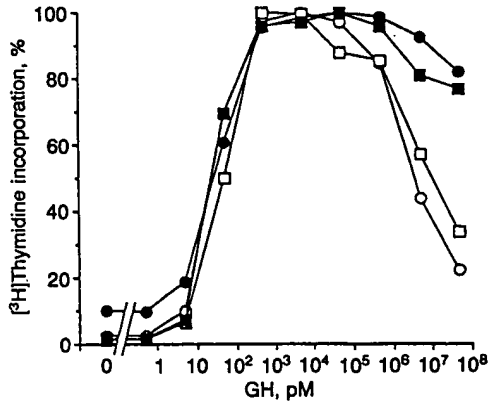

FIG. 5. Effect of GH on DNA synthesis of FDC-P1 transformants expressing chimerae. The FDC-P1 transformants expressing HC2 (○), RC3 (●), RC4 (□), and RC5 (■) were cultured with 0–50 μM human GH. [3H]Thymidine incorporation is presented as percentage maximum incorporation observed with an optimal concentration of GH for each transformant.

tionally interchangeable with respect to transducing growth signal in FDC-P1 cells.

DISCUSSION

In this study, we established cell lines derived from an IL-3-dependent mouse myeloid precursor FDC-P1 cell line and that constitutively express GH-R or GH-R/G-CSF-R chimerae. GH stimulates chondrogenesis and myogenesis *in vivo* (11). It also stimulates the DNA synthesis of chondrocytes and induces the differentiation of preadipocytes into adipocytes *in vitro* (11). These functions and the target cell specificities are quite different from those of G-CSF and IL-3. Nevertheless, GH-R could transduce a growth signal in IL-3-dependent FDC-P1 cells. Since the cytoplasmic region of GH-R is essential for GH-R-mediated growth signal transduction (P. Colosi and W.I.W., unpublished results), this result suggests that the molecule interacting with GH-R in the cytoplasm may be identical or similar to those used for IL-3 and G-CSF receptors. In this regard, it may be notable that GH and IL-3 induce phosphorylation of the respective receptor at tyrosine residues (23, 24). Similar tyrosine kinases may be involved in growth signal transduction of the GH, IL-3, and G-CSF systems.

The transformant cell lines established in this report will provide a simple and sensitive assay system for biologically active GH. Furthermore, the transformant cell lines responsive to GH for proliferation will help to elucidate the signal transduction mechanism mediated by GH-R. Previously, we have shown that transformed cell lines expressing the HC2 chimera respond to GH only at the concentration of GH that induces dimerization of the receptor (20). In this report, this finding was confirmed with transformed cell lines expressing rabbit GH-R and other chimerae with G-CSF-R. At higher concentrations of the ligand where no dimerization of the extracellular domain of the receptor occurs (16), neither rabbit GH-R nor the chimeric molecules could transduce the growth signal, suggesting that ligand-induced dimerization of the receptor is necessary for signal transduction. Since the chimerae carry the cytoplasmic region of G-CSF-R, these results may support the notion that G-CSF-R works as a homodimer like GH-R. Dimerization of the receptor should induce association of the cytoplasmic regions, which may be a crucial step to generate a site of interaction with intracellular substances or effectors.

To study the functions of proteins such as enzymes, cytokines, and their receptors, various hybrid proteins have been prepared by recombinant DNA technology (25–28). The most common method of preparing hybrids is to construct a chimeric cDNA by using appropriate restriction enzyme recognition sites and to express it in *E. coli* or mammalian cells. However, this method often introduces deletions or insertions of several amino acids at the junction of the hybrid, which may result in an inappropriate conformation. G-CSF-R and GH-R have a mosaic structure consisting of subdomains such as CRH, transmembrane, and cytoplasmic regions. Each subdomain of G-CSF-R and GH-R is encoded by sets of exons with a similar organization. Therefore, we constructed chimeric G-CSF-R and GH-R by exon swapping using recombinant PCR. Except for the RC1 construct, all the chimeric molecules transduced the growth signal into cells, indicating that the transmembrane domain and the cytoplasmic region of G-CSF-R and GH-R are functionally interchangeable despite the little similarity between them. The exon-swapping method we described will therefore be applicable to construction of various hybrids, even those consisting of unrelated molecules.

The RC1 chimera is a hybrid in the CRH domain, which can be divided into two subdomains of ≈100 amino acids (5, 7, 21). Although the N-terminal subdomain of G-CSF-R plays a major role in binding the ligand, the C-terminal subdomain was also indispensable for constituting a high-affinity binding site for G-CSF (9). Within GH-R, several amino acids such as Glu-127, Trp-169, and Asn-218 in the C-terminal region of the CRH domain interact with GH (15). The inability of the RC1 chimera to bind GH may be explained by the fact that these amino acids in the C-terminal region of the CRH domain of GH-R are not conserved in G-CSF-R. The interchangeability of the transmembrane domain and the cytoplasmic region between G-CSF-R and GH-R indicated a similar signal transduction system for G-CSF-R and GH-R. As discussed above, G-CSF and GH have differentiation-inducing activities in addition to growth-promoting activity. G-CSF induces differentiation of neutrophilic granulocytes (1, 2), while GH induces differentiation of adipocytes (12). Whether or not these differentiation signals are mediated by similar mechanisms in G-CSF-R and GH-R remains to be elucidated.

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